The Effect of Heavy Metal Pollution in Aquatic Environments on Metallothionein Production in *Mytilus sp.*

By

Lee Ann Acker, Jeremiah R. McMahan, and James E. Gawel

Keywords: metallothionein, metal, Puget Sound, mussel, Mytilus edulis, cadmium

Abstract

Industrial development in Puget Sound has resulted in heavy metal contamination of local waters. Ecosystem contamination from cadmium, copper, and lead pollution may damage marine organisms at the cellular level and possibly affect the ecological balance. Metallothionein (MT) is a low molecular weight protein that binds heavy metals in marine organisms. This makes MT a valued *in situ* biomarker of heavy metal pollution in aquatic environments. In this study, we examine the temporal and spatial changes in MT production in *Mytilus sp.* populations growing on floating docks in south and central Puget Sound. MT is also measured in transplanted common-stock *Mytilus sp.* at the same sample sites to address possible heavy metal resistance through adaptation. We compare MT concentrations to metal levels in the water column and mussel tissues.

Introduction

Point and non-point sources of trace metals are evident in Puget Sound waterways in Washington State. Steady population growth and industrialization in the Puget Sound area have lead to an increase in metal pollutants in the sediments and water column (Paulson and Freely 1985). The major anthropogenic sources of heavy metals in Puget Sound are industrial and urban waste, wastewater discharges and shipping activity (Lionetto et al. 2001). Of the Sound's 1.8 million submerged acres, 5700 acres of highly industrialized areas are highly contaminated (Puget Sound Action Team 2004). Although recent surveys demonstrate a gradual decline in heavy metals in Puget Sound over the last decade (Bolton et al. 2003), their presence has triggered fish and shellfish consumption advisories over the years (Puget Sound Action Team 2004). Exposure and ingestion can cause health problems in people and animals including neurological and reproductive problems.

Measuring heavy metals in aquatic organisms may be a bioindicator of their impact on organism and ecosystem health (Krishnakumar et al. 1994), but a true evaluation of the damage inflicted by heavy metals should come from comprehensive biomarker studies. Biomarkers are more telling than bioindicators as measurements of heavy metal contamination because they deal with chemical and physiological changes on the organism level and assess contamination based on a direct measure of change in the organism (Lafontaine et al. 2000, Allen and Moore 2004). Research over time has focused on various species and various biomarkers to determine the amount of heavy metal toxicity in aquatic environments. Albaugh (2002) used sea anemones, Aspholm and Hylland (1998) used sea urchins, Downs et al. (2001) used grass shrimp, and Regoli et al. (2002) used a benthic fish called the red mullet. Biomarkers in mussels such as glutathione (GSH) and metallothionein are often used to evaluate heavy metal contamination (Rainbow 2000).

Metallothionein (MT) is a low molecular weight, cysteine-rich protein that binds 7 moles of cadmium or zinc to every one mole of MT (Klaassen et al. 1999). The primary purpose of metallothionein in cells is to regulate copper and zinc homeostasis and to detoxify the cell of cadmium and mercury (Klaassen et al. 1999). Overexposure to heavy metal contaminants can lead to overproduction of MT and consequently systemic damage to the organism (Krishnakumar et al. 1994, Lowe 1998, Ringwood et al. 2004, Petrovic et al. 2001, and Cavaletto et al. 2002). Although many species produce metallothionein and can be tested for metal toxicity via MT measurements, mussels have demonstrated higher rates of accumulation for metals than other species because of their filter feeding and sessile life histories. This has been shown to be especially true for cadmium (Kavun et al. 2002). However, previous experiments with MT and metal contamination fail to address the possibility of organism adaptation to a polluted environment and resulting alterations in MT production (Mouneyrac et al. 2002).

Mytilus edulis, the original species recognized in Puget Sound, has now been separated into two genetically distinct populations. A northern type designated *Mytilus trossulus* and a southern type, *Mytilus galloprovincialis* (Nybakken 1995). These two species tend to hybridize where both exist and can only be differentiated by genetic testing (Nybakken 1995). Shell size and shape play a role in indicating species, but this method is not completely accurate (Innes and Bates 1999). In this study, we examined *Mytilus* mussels of similar size, weight and shape to minimize species differences in our results (Duquesne et al. 2004)

The purpose of this study was to compare MT levels in the mussel digestive glands to the levels of lead, copper, and cadmium in mussel tissues and their waters of origin to determine the efficacy of using MT as a biomarker for heavy metal stress. Mussels were tested at seven locations in south and central Puget Sound. To address the question of mussel adaptation to metal pollution (Roesijadi 1992), we transplanted farm-raised mussels from a common source to each sampling site and tested them before and after the transplant.

Materials and Methods

Sample Collection Methods

Seven sites were selected in south and central Puget Sound. Site locations were Hylebos (HYL), Thea Foss (TF), Old Town, Commencement Bay (OT), Point Defiance (PD), Fox Island (FOX), Port Orchard (PO), and Olympia, Budd Inlet (OLY) (Figure 1). Water temperature, dissolved oxygen, pH and specific conductivity/salinity were measured in the field using a hand-held probe. Five mussels were harvested from the side of floating docks at each site. The mussels collected were 50-75 mm in length. Water samples from each site were collected for nutrients, metals, and chlorophyll analyses. Samples were kept on ice during transport. Mussels were dissected in the laboratory the same day. Digestive glands were removed and frozen in labeled vials in liquid N_2 prior to analysis for MT. The remaining mussel tissue was placed in a drying oven at 60°C for five days. Because of the time involved in sampling and dissection the sites were separated into two groups sampled on alternate weeks (group 1: OLY, FOX, and PO; group 2: HYL, TF, OT and PD). For the transplant experiment, we placed five mussels from common stock (Taylor Shellfish) at each of our sample sites. Metal and MT levels were tested in the soft tissue and digestive glands respectively after 20 days and compared to levels in non-transplanted mussels as a control.

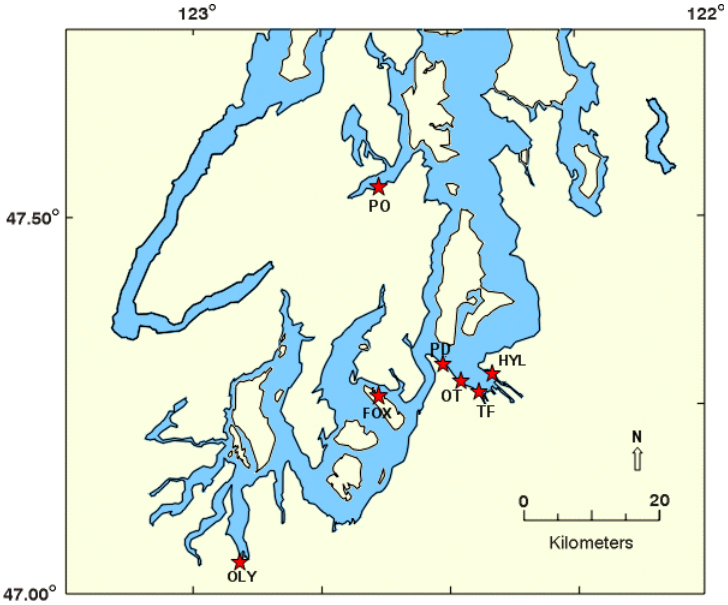

Figure 1: Map of Puget Sound sampling sites included in this study.

Metals Analyses

Dry mussel bodies were homogenized with a ceramic mortar and pestle and microwave (MARS 5, CEM) digested in Teflon containers in nitric acid. Digested mussel tissues were tested for lead, copper and cadmium concentrations via graphite furnace atomic absorption spectrometer. Unfiltered water samples were sent to the Washington State Department of Ecology's Manchester Environmental Laboratory where they were analyzed for cadmium, lead, and copper.

Nutrient Analysis

Water samples were collected and filtered on site through a 0.45 µm syringe filter and kept in the freezer prior to analysis. Samples were tested for phosphate, silicate, nitrite, nitrate, and ammonium by the University of Washington Marine Chemistry Laboratory using a Technicon Model AAII.

Chlorophyll

We tested for chlorophyll using water samples filtered onto glass fiber filters and sonicated with 90% acetone for 7 minutes. The samples were then centrifuged for 5 minutes at 3700 rpm. The supernatant was analyzed in a Turner Design TD-700 fluorometer.

Metallothionein Analysis

We analyzed mussel digestive glands for metallothionein using a modification of the method described by Viarengo et al. (1997). Samples were homogenized in a buffer solution of 0.5 M sucrose, 20 mM Tris-HCL (pH 8.6), 0.006 mM leupeptin, 0.5 mM PMSF, and 0.01% ß-mercaptoethanol with a PT 2100 (Brinkmann) laboratory mixer then centrifuged for 20 minutes at 30,000 g at -4ºC. The supernatant of that sample was purified with 1.05 mL chilled (on ice) ethanol and 80 µL chloroform and centrifuged for 10 minutes at 6000 g at -4ºC. We then added 40 µL concentrated HCL and 6 mL cold ethanol and allowed the proteins to denature for one hour at -20°C. This was then centrifuged for 10 minutes at 6000 g at -4ºC and the pellet saved. We did not add RNA in the MT pelletization step as this has been shown to be unnecessary (Viarengo et al. 1997). We discarded the supernatant and added 1 mL of the previously described buffer solution, 6 mL cold ethanol and 80 µL chloroform and centrifuged for 10 minutes at 6000 g at -4ºC. We discarded the supernatant and dried the pellet with N_2 gas. We resuspended the pellet with 150 µL 0.025 M NaCl and 150 µL 1 N HCL with 4 mM EDTA. After resuspension, we added 4.2 mL of a solution containing 2 M NaCl and 0.43 mM DTNB and 0.2 M NaH_2PO_4 (pH 8.0), and centrifuged for 5 minutes at 2500 rpm. The supernatant was analyzed at 412 nm in a spectrophotometer. Results were compared to a GSH calibration curve.

Results and Discussion

Metal Concentrations in Water

Total metal concentrations in surface seawater samples varied greatly from date to date. All Cd concentrations were below detection limits. Copper and lead concentrations increased significantly on the last sampling date (Figures 2 and 3). This rise could be due to a significant runoff event initiated by a storm that occurred the day before sampling (USGS 2005). These elevated levels would not have had time to influence copper levels or MT levels in mussels sampled that day. Runoff from the Puyallup River affects the HYL, TF, PD, and OT sites. All other sampling days show much lower levels of lead and copper. Unfortunately, the separation of sampling sites into two groups sampled on alternate weeks makes comparison among all sites difficult. However, it is obvious that higher levels of lead and copper are found at the Hylebos Waterway (HYL) and Budd Inlet (OLY) locations when compared to the other sites sampled on the same days. This may result from the direct input of metals from Hylebos Creek and the Deschutes River at these respective sites.

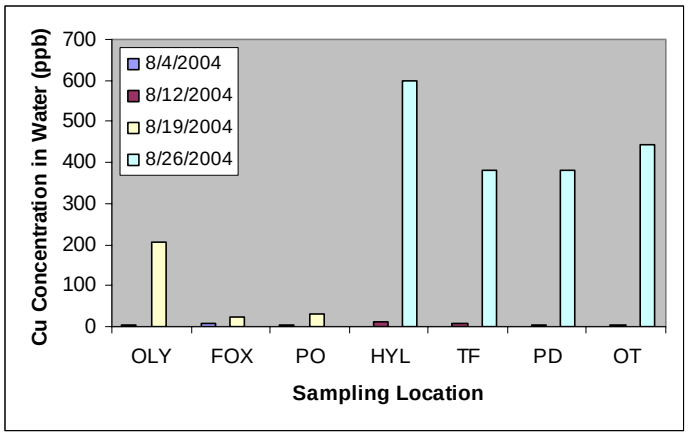

Figure 2: Copper concentrations in surface water samples. OLY, FOX, and PO samples were collected on 8/4 and 8/19, while HYL, TF, PD, and OT samples were collected on 8/12 and 8/26.

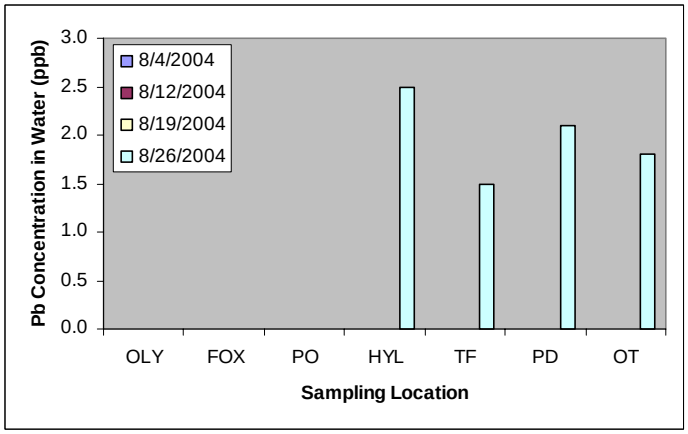

Figure 3: Lead concentrations in surface water samples. OLY, FOX, and PO samples were collected on 8/4 and 8/19, while HYL, TF, PD, and OT samples were collected on 8/12 and 8/26. Samples not shown are below detection limit.

Metal Analysis in Mussel Tissue

Although variability was quite high from mussel to mussel, the highest concentrations of Cd, Cu, and Pb were consistently found at the Thea Foss Waterway (TF) and Point Defiance (PD) locations (Figures 4,5, and 6). In addition, higher Cu levels were also found in mussels at the Port Orchard (PO) site in Sinclair Inlet, and higher Pb levels were found at the Hylebos Waterway (HYL) site. The PD site is located adjacent to the site of the former ASARCO smelter and current Superfund site. The TF and HYL sites are also part of the same Superfund site (Commencement Bay Nearshore/Tideflats). Although the PD, TF, and HYL locations are undergoing environmental cleanup, higher levels of cadmium, lead, arsenic and copper have been found in the sediments and water column within the Superfund site boundaries. The PO site is located near the Puget Sound Navel Shipyard where copper antifouling agents may be elevated in the sediments and water column. Copper and cadmium are consistently low and lead is below detection limits at the OLY, FOX, and OT sites.

Metallothionein Production

While metal concentrations in mussel tissues are consistent with known sources of metal contamination in south and central Puget Sound, metallothionein levels in the digestive glands of these mussels are not (Figure 7). MT concentrations are relatively uniform at all sampling sites. Especially notable is the fact that some of the lowest MT levels are detected on August 26, when high levels of Pb and Cu were detected in water samples, and some of the highest levels of all metals were measured in mussel tissues. Therefore, the results of our study suggest no connection between Pb, Cu, and Cd contamination and metallothionein production in mussels in south and central Puget Sound.

Transplant Analysis

The Cu and Cd concentrations in the control mussels were equal to or greater than mussels placed at the sampling sites, and we found no significant differences between metal concentrations at any of the sampling sites (Figure 8). Furthermore, with the exception of the PO site, metallothionein levels are uniform at all locations (Figure 9). As metal concentrations were higher in mussels prior to transplanting, it is difficult to interpret the results of this experiment. We again see no correlation between metal concentrations in mussel tissue and metallothionein production.

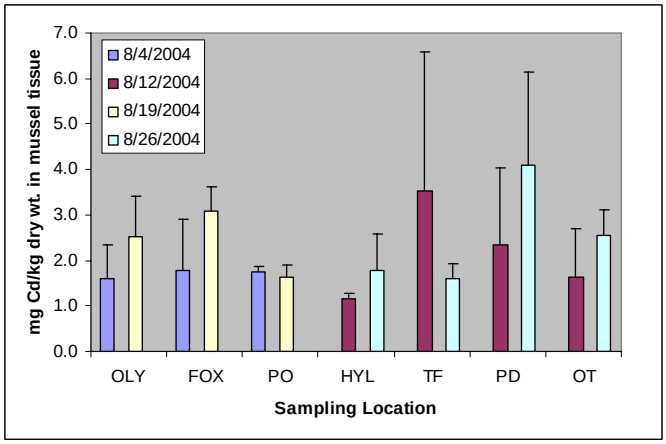

Figure 4: Cadmium levels in mussel tissues. Error bars are one standard deviation from the mean (n=2).

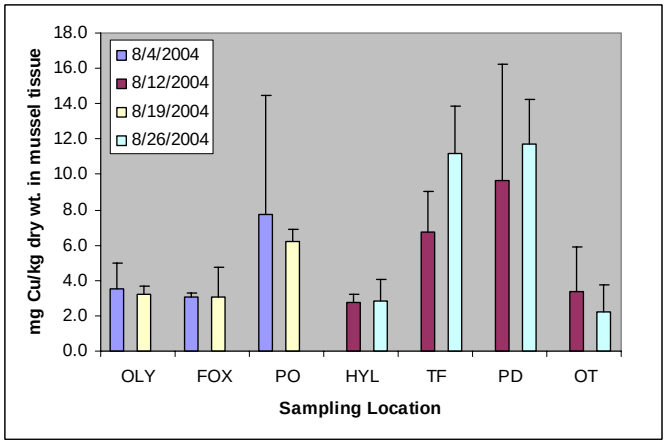

Figure 5: Copper levels in mussel tissues. Error bars are one standard deviation from the mean (n=2).

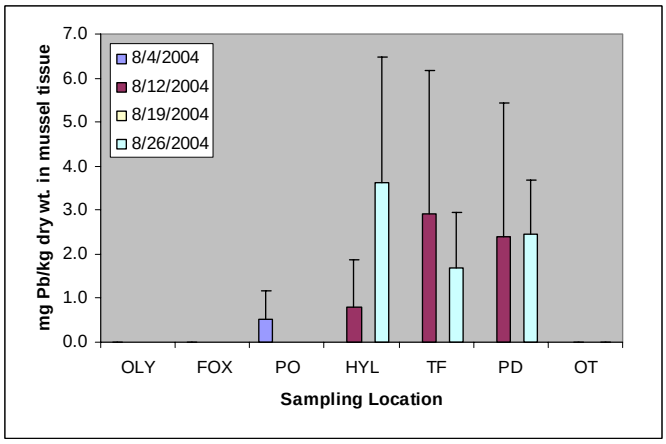

Figure 6: Lead levels in mussel tissues. Error bars are one standard deviation from the mean (n=2).

In addition, the pattern of metallothionein production in our transplant experiment differed from our *in situ* results (Figure 7).

Conclusion

Our results suggest either that MT production in mussels may not be an effective biomarker for metal stress or that the metal concentrations measured may not be high enough to induce a metal stress response in these organisms. This could be due to mussels having a high tolerance to metal contamination, making them an undesirable biomarker species. Mussels may also have other mechanisms to deal with higher levels of metals, such as glutathione, making metallothioneins non-indicative of metal stress.

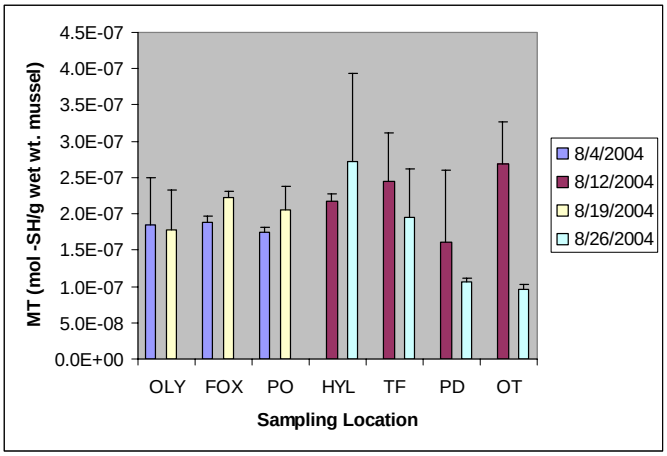

Figure 7: MT levels in mussel tissue. Error bars are one standard deviation from the mean (n=2).

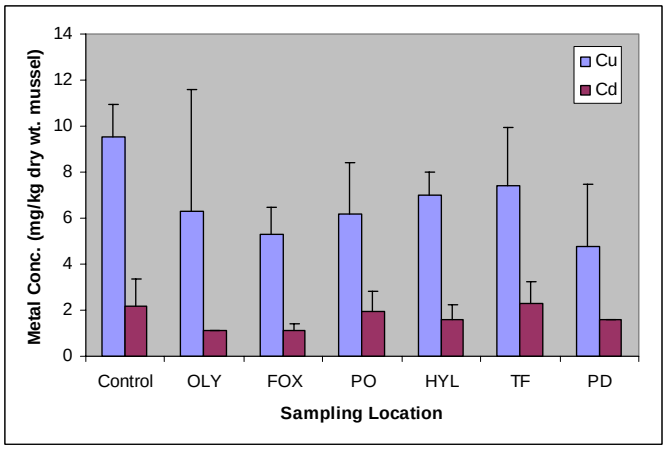

Figure 8: Metal concentrations in transplanted mussels and controls. Error bars are one standard deviation from the mean (n=2).

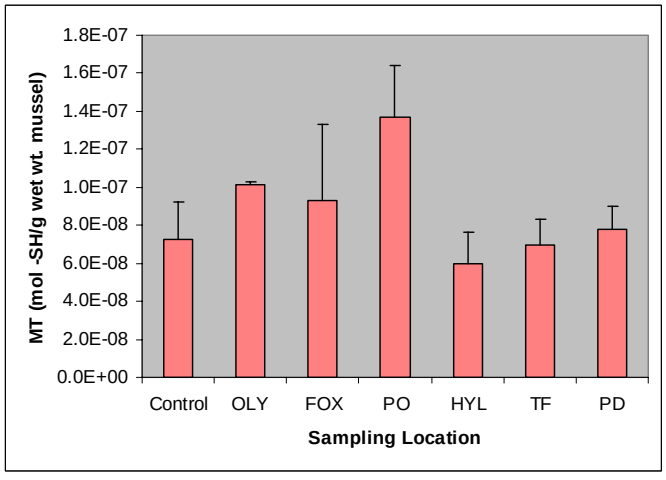

Figure 9: Metallothionein concentrations in transplanted mussels and controls. Error bars are one standard deviation from the mean (n=2).

In addition, cadmium has been shown to be the primary target for sequestration by metallothionein (Klaassen et al. 1999) but cadmium levels in the mussels collected in our study were not significantly different at any of the sampling sites (Figure 4). Therefore if cadmium concentrations control the metal stress response in mussels and no significant variations in cadmium levels were present at our sampling sites, then uniform MT concentrations would be expected.

Future studies of metal stress in the Puget Sound ecosystem may need to examine other indicator species and other biomarkers. As metal pollutants accumulate in Puget Sound sediments, using a benthic organism as an indicator species may be particularly useful for assessing toxicity. In addition, other biomarkers of metal stress exist. For example, Petrovic et al. (2001) studied lysosomal membrane destabilization in *Mytilus sp.*, *which* occurs upon initial exposure to metal contaminants prior to the induction of an MT response.

Future transplant experiments should use organisms from a known low-metal location and should include pre-placement equilibration in clean medium in the lab. Sampling transplanted organisms on the same dates as local populations would also facilitate interpretation of the results of this experiment.

Acknowledgements

Funding for this project was provided by a University of Washington Tacoma Founders Endowment Grant. We would also like to thank Joel Elliott and the University of Puget Sound Biology Department for access to their instrumentation. Mussels for the transplant experiment were provided by Taylor Shellfish, Shelton, Washington.

Works Cited

Albaugh, A., 2002, Metallothionein analysis as a measure of heavy metal stress in the sea anemones *M. senile, A. elegantissima,* and *A. Artemisia* in Puget Sound, WA, *Unpublished research, Civil and Environmental Engineering, University of Washington*, June 21.

Allen, J., and M. Moore, 2004, Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation?, *Marine Environmental Research*, **58**:227-232.

Aspholm, O., and K. Hylland, 1998, Metallothionein in Green Sea Urchins (*Strongylocentrotus droebachiensis*) as a biomarker for metal exposure, *Marine Environmental Research*, **46**(1-5):537-540.

Bolton, et al., 2003, Organic and trace metal contaminants in sediments and English sole tissues from Vancouver Harbor, Canada, *Marine Environmental Research*, **57**:19-36.

Cavaletto, M., et al., 2002, Effect of hydrogen peroxide on antioxidant enzymes and Metallothionein level in the digestive gland of *Mytilus galloprovincialis*, *Comparative Biochemistry and Physiology*, **131**(C):447-455.

Downs, C., et al., 2001, Assessing the health of Grass Shrimp (*Palaeomonetes pugio)* exposed to natural and anthropogenic stressors: A molecular biomarker system, *Marine Biotechnology*, **3**:380-397.

Duquesne S. et al., 2004, Sub-lethal effects of metal exposure: Physiological and behavioral responses of the estuarine bivalve *Macoma balthica*, *Marine Environmental Research*, **58**: 245-250.

Innes, D.J., and J.A. Bates, 1999, Morphological variation of *Mytilus edulis* and *Mytilus trossulus* in eastern Newfoundland, *Marine Biology*, **133**:691-699.

Kavun, V.Y., et al, 2002, Metal accumulation in mussels of the Kuril Islands, northwest Pacific Ocean, *Marine Environmental Research*, **53**:219-226.

Klaassen, C., et al, 1999, Metallothionein: An intracellular protein to protect against cadmium toxicity, *Annu. Rev. Pharmacol. Toxico*, **39**:267-294.

Krishnakumar P.K. et al, 1994, Effect of environmental contaminants on the health of *Mytilus edulis* from Puget Sound, Washington, USA. I. Cytochemical measures of lysosomal responses in the digestive cells using automatic image analysis, *Marine Ecology Progress Series*, **106**:249-261.

Lafontaine,Y. et al, 2000, Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada), *Aquatic Toxicology*, **50**:51-71.

Lionetto, M.G. et al, 2001, Biomonitoring of heavy metal contamination along the Salento coast (Italy) by metallothionein evaluation in *Mytilus galloprovincialis* and *Mullus barbatus*, *Aquatic Conservation: Marine and Freshwater Ecosystems*, **11**:305-310.

Lowe, D., et al, 1995, Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study, *Marine Ecology Progress Series*, **129**:189-196.

Mouneyrac C., et al, 2002, Partitioning of accumulated trace metals in the talitrid amphipod crustacean *Orchestia gammarellus*: a cautionary tale on the use of metallothionein-like proteins as biomarkers, *Aquatic Toxicology*, **57**:225-242.

Nybakken, James, 1995, *Diversity of the Invertebrates: A laboratory Manual Pacific Coast Version*, McGraw-Hill, 328pp.

Paulson, A.J., and R.A. Feely, 1985, Dissolved trace metals in the surface waters of Puget Sound, *Marine Pollution Bulletin*, **16**(7):285-291.

Petrovic, S., et al, 2001, Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis Lam.*) as biomarkers in a field study, *Marine Pollution Bulletin*, **42**(12):1373-1378.

Puget Sound Action Team, 2004, *State of the Sound*, Office of the Governor, Olympia, WA, 62pp.

Rainbow, P.S., et al, 2000, Biomonitoring of trace metals in the Gulf of Gdansk, using mussels (*Mytilus trossulus*) and barnacles (*Balanus improvisus*), *Water Resources*, **34**(6):1823-1829.

Regoli, F., et al, 2002, Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet, *(Mullus barbatus)*, *Marine Pollution Bulletin*, **44**:912-922.

Ringwood, A.H., et al, 2004, Linkages between cellular biomarker responses and reproductive success in oysters – *Crassostrea virginica*, *Marine Environmental Research*, **58**:151-155.

Roesijadi, G., 1992, Metallothioneins in metal regulation and toxicity in aquatic animals, A*quatic Toxicology*, **22**:81-114.

USGS, 2005, Daily Discharge CFS for Puyallup River, *USGS Water Resources*, http://nwis.waterdata.usgs.gov, accessed 3/26/05.